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November 16, 2004

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APPLICATION NUMBER: *60/508,660*
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Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office

TITLE:

"A method to biophysically- and genetically-engineer human endothelial cells in vitro to express functionally normal intercellular communication for seeding onto implantable endovascular devices."

DESCRIPTION:

#11 in Disclosure Form: Description, supporting data, sifgnificance, novelty, etc:

Summary of the invention:

We propose a method/technology to generate Human Endothelial Cells in vitro with the functional intercellular communication that is consistent with a normal cell phenotype in vivo. These cells are generated to be used in the engineering of functional cardiovascular constructs (e.g. vacular grafts, heart valves) and other vascular implants (e.g. stents) that benefit from having an antithrombotic and proactive surface only attainable with a fully functional endothelial lining. Specifically, we propose a method to achieve functional communication in otherwise communication-impair cells by inducing the expression and organization of the gap junctional protein Cx40, a key protein for intercellular cellular communiaction and normal function in human vascular tissue. Our proposed method combines biophysically- and genetically-engineering approaches to achieve the desired results. The biophysical approach is based on fluid flow stimulation of the production and organization of the Cx40 protein. We have demonstrated the feasibility of this approach (see attached supporting data) and found that the expression of the protein and functional communication associated with the stimulus is time and flow magnitude dependent. This allows for a precise regulation and control of the desired properties of the culture cells. The genetically-engineering approach is based on the fact that gene transcription can be regulated by the insertion of a Cx40 gene promoter in human cells in culture. This normal endothelial gene can be either under the control of a promoter that is constitutively active or under the control of a regulable promoter that can be turned on and off to regulate gene transcription as desired. This is achieved by the administration of Tetracyclin (Tat-on, Tat-off promoters). Both approaches described above are very powerful in the regulation of Cx40 expression. Nevertheless, we proposed that is the proper combination of this two approaches what will result in an optimal cell product for implantation. These are cells that a) possess the appropriate type and level of intercellular communication, and b) are readily adapted to the natural flow dynamic environment that they will encounter after implantation.
The control regulation of Cx40 expression and organization that we expect to obtain with the combined genetically- and biophysically-engineering approach will result in an intercellular communication phenotype that will resemble the natural tissue and should be consistent with a normal function of the endothelium making these cells suitable and more competent than regular culture cells for use in vascular implants.

Rationale and Background Information:

Endothelial intercellular communication occurs through Gap Junctions, connexin protein channels that allow the direct cell-cell transfer of ions and small signaling molecules. Endothelial Gap Junctional Intercellular Communication (GJIC) plays an important role in vascular tissue homeostasis including the coordination of cell growth and migration, vascular vasomotor responses, and angiogenesis (Ross, R.,1995, Ann Rev Physiol; 57: 791-804, Chaytor A.T, et al., 1998, J. Physiol 508, 2:561-573).
Gap junctions establish homotypic (endothelial/endothelial) and heterotypic (endothelial/smooth muscle, endothelial/circulating leukocyte) communication pathways that are essential for the maintenace of normal vascular function. Gap junctions are important in coordinating endothelial cell migration and replication during wound repair after denudation and during angiogenesis and in the propagation of signals up and down the length of the vessel to regulate blood flow through endothelium-mediated vasoregulation. The importance and extreme efficiency of GJIC between endothelial cells in vivo is well established (Segal S.S., and Beny, J.L. Am J Physiol, 1992).

One of the greatest challenges in the engineering of vascular grafts is to be able to reproduce the sofisticated functions of a natural endothelial layer. To that end, efforts of the last decade have been focused on including the use of endothelial cells in the engineering of vascular grafts. These approaches involve the use of endothelial cells that have been harvested from natural tissue, followed by the culture/amplification, and seeding of these cells on natural matrices or polymeric substrates. Unfortunatelly, the succes of this approach has been modest. It has been reported that upon implnatation and restoration of physiological flow and pressure conditions, the seeded cells are lost from the surface of the graft. We hypothesize that GJIC is critical to endothelial cell coordinated adaptation and that endothelial cell adhesion to its substrate is one of those cellular functions dependent on functional GJIC. In particular during early stages of endothelial layer remodeling and adaptation to a physiological flow environment. Therefore, the lack of proper cell communication that we have recently demonstrated in cultured human endothelial cells may explain why endothelial cells seeded on vascular graft material have consistently come off the substrate upon restoration of physiological flow conditions (constructs implanted in animal models).
Once removed from their natural environemnet endothelial cells are known to loose some of their functional properties. Our recent studies have demonstrated that intercellular gap junctional communication in Human Aortic Endothelial Cells is severly impaired once cells are removed from the natural vessel and set in in vitro culture conditions.
Knowing the importance of endothelial intercellular communication in maintaining vessel function it becomes a must to restore communication to cells on a vascular construct prior to implantation or use cells of functional level of communication in the engineering of these vascular constructs.

Vascular endothelial cells in vivo are immunopositive for Cx40, 43, and 37 although it is thought that in humans gap junctional intercellular communication (GJIC) is mediated primarily by Cx40 with lower expression of Cx43 and Cx37 (Bastide, B., et.al., Circ Res, 1993; Bruzzone, R., et.al., Mol Biol of the Cell, 1993). However, it is well established that in culture, Cx43 is the most prominent, and perhaps the only connexin expressed

(Pepper, M.S., Am J Physiol, 1992). As supporting data for this invention we have demonstrated that cultured Human Aortic Endothelial Cells (HAEC) are able to reach functional level of communication comparable with that of natural tissue by regaining expresssion of Cx40 and Cx37. We achieved this functional level by exposing the cultured cells to controled fluid flows of physiologically relevant characteristics (see attached figures summarizing these findings). As outlined in the summary above we proposed to biophysically- and genetically-engineer human endothelial cells seeded onto implantable endovascular devices to express Cx40 and achieve a normal intercellular communication previous to implantation.

Our laboratory has previosly demonstrated and published (DePaola, N., et.al. (1999). Proc. Natl. Acad. Sci., 96:3154-3159) the ability to regulate Cx43 expression with flow in animal cells. Recent studies have also demonstrated that hemodynamics alters gap junctional intercellular communication (GJIC) and Cx43 protein expression in in vivo animal models (Gabriels, J.E. and Paul, D.L. (1998). Circ. Res., 83:636-643). Nevertheles, the regulation of Cx40 and/or Cx37 by flow in Human cells and the fact that levels of communication and protein expression and distribution that resemble functional conditions in natural tissue can be achieved in vitro has not been previosuly demontrated. The concept of using this approach to generate functionally conpetent cells in the engineering of vascular garfts and implantable endovascular devices is new.
We have no knowlegde of any strategy or effort to geneticaly-engineer human endothelial cells for Cx protein expression and organization to generate functionally conpetent cells in the engineering of vascular garfts and implantable endovascular devices. This concept is new.

#12 in Disclosure Form: Current Status, Research Effort required, etc:

Vascular gap junctions are formed by the connexin proteins Cx37, Cx40 and Cx43. These proteins are very dynamic exhibiting rapid turnover times and variable expression patterns. Different channel properties are associated with Cx type. In humans gap junctional intercellular communication (GJIC) is mediated primarily by Cx40 with lower expression of Cx43 and Cx37.
To date, we have investigated the dynamic regulation of endothelial GJIC by fluid flow and the role of each type of functional connexin channels in intercellular communication in human endothelial cells. Specifically, we evaluated alterations in functional communication and vascular gap junction protein expression and organization in human aortic endothelial cells exposed to physiologically relevant flows in vitro.

Data supporting this invention has been generated and it is attached to this document. In brief, these studies demonstrated a dynamic regulation of all vascular connexins by flow, in human aortic endothelial cells. Flow increases functional gap junctional communication by de novo expression of Cx40 protein and its assembly in functional channels. Inhibition studies revealed a contribution of Cx37, but to a lesser extent. The role of Cx43 in flow-induced communication is negligible. This is consistent with what is observed in vivo in normal tissue.

We investigated three fluid shear magnitudes: 0, 2.5, and 12 dynes/cm2 and three time period for flow exposure: 6, 16, and 24 hours.
Results from Functional Intercellular Communication evaluated by dye injection (Lucifer Yellow, MW 476) demostrated that Cell-cell communication is significantly increased with flow exposure (1.8-, 3.5-, and 7.5-fold increased with 5, 16 and 24 hours in flow, respectively) Data was obtained from 16 independent experiments, 7 individual cell injections were performed in each monolayer studied.
The evaluation of protein expression by Western blot analysis demonstrated that Control monolayers are abundant in Cx43 with low expression of Cx40. Upon exposure to flow Cx40 protein expression is significantly increased while Cx43 is moderately regulated.
Protein Localization studies by Immunocytochemistry using specific antibodies for Cx40 (Chemicon) and Cx43 (Zymed, Chemicon), revealed that Control (no-flow) monolayers are abundant in Cx43 localized at cell borders while Cx 40 is scarce/rare. After 24 hrs of flow Cx40 immunofluorescence increases and it is found at cell-cell appositions. Cx43 is observed/found in intracellular compartments along with decreased immunoreactivity at cell borders.
The evaluation of the Specificity of Functional Channels using connexin-mimetic peptides to block Cx43, Cx40, and Cx43 and Cx37 simultaneously (Sigma Genosys) was performed by treating the monolayers with the connexin-mimetic peptide inhibitors followed by dye injection to evaluate extent of cell-cell communication. Results demonstrated that 16-hr of flow exposure increased dye transfer 3.5-fold compared to no-flow controls. Post-flow incubation with connexin43-mimetic peptide did not affect dye-coupling, while Cx40 peptide indicated significant decrease (57%) of dye-coupling. Simultaneous blocking of Cx37 and Cx43 channels decreased flow-induced dye-coupling by only 26%, which is attributed to Cx37 since Cx43 inhibition did not result in a reduction of cell communication.

Ongoing research efforts:

For the genetic-engineering approach, we'll try couple of different promoters:
1) Constitutively active promoter---always active. We will engineer the cells by inserting a normal endothelial gene that is under the control of a constitutively active promoter so cells will immediately make the desired Cx40.
2) Tat-on, Tat-off promoter that can be regulated by the administration of Tetracyclin This promoters respond in a dose dependent manner to tetracycline treatment, so by controlled administration of tetracycline the rate of transcription of the Cx40 gene can be regulated.

We have already have found that the expression of Cx40 is flow sensitive. Therefore, function on the cultures is expected to be turned on with flow. The advantage of the insertion of the Cx40 gene promoter is that expression of Cx40 will occur right away. The advantage of combining the two approaches biophysically- and genetically-engineered cells is that it is expected that other important functions are restored by the exposure to the physiologically relevant flow environment via adaptation to a new

dynamic condition that will promote and aid integration of the engineered construct with the natural surrounding tissue after implantation.

Once GJIC regulation in flow and/or gene insertion is identified, we will correlate with selected changes in cell functions (alignment with flow, proliferation, migration, cell loss, apoptosis and modulation of intercellular adhesion molecules) in an effort to link gap junction regulation to cell functions relevant to vascular homeostasis

Communication is normally at high levels in native tissue. Endothelial cells function better once they are together as one "continuous" layer. Cells that have been gentically engineering to expressed Cx40 will be seeded in monolayers before being exposed to controled flow stimulation for further regulation of cell communication and other key cell functions. Prolonged exposure to physiological flow (biophysically-engineering approach) is known to increase intercellular adhesion and aid in the formation of a tight endothelial barrier (a key function of naturally functional endothelium).
Cells genetically engineered to expressed Cx40 are expected to be more readily responsive to the flow stimulus making the combination of the two proposed approaches a more efficienta and effective way to manipulate the final characteristics of these engineered monolayers linning the constructs for implanatation.

As we treat the culture cells with flow and gene insertion we'll evaluate key endothelial functions to acces the level of functionally acquire from the treatment and predict the potential success of a vascular construct that will contain these cells and specific treatment. Various byophysical parameters (flow magnitudes and time of exposure) will be explored. Inhibitor peptides will be used after flow conditioning and/or gene insertion to determine which endothelial functions are closely associated with the genetically- and/or biophysically-engineered cultures.

Engineered cells seeded on substrates and matrices commonly used in tissue engineering of vascular grafts will be exposed to physiological challenges in vitro in our laboratory. This challenges include the simulation of hypertension, hypercholesteremia , and other risk factors present in patients of compromised vascular vessel function since these are the patients likely to host an engineered vascular graft (and other endovascular devices).

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

A method to biophysically-and genetically-engineer human endothelial cells in vitro to express functionally normal intercellular communication for seeding onto implantable endovascular devices

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ENCLOSED APPLICATION PARTS (check all that apply)

☑ Specification Number of Pages 5
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☐ Drawing(s) Number of Sheets
☐ Other (specify)
☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.
☑ A check or money order is enclosed to cover the filing fees
☐ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:
☐ Payment by credit card. Form PTO-2038 is attached.

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.
☑ Yes, the name of the U.S. Government agency and the Government contract number are: NIH - HL64728

Respectfully submitted,

SIGNATURE _____ Date 10/3/03

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Docket Number: RPI-841

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

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